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TI Structure-activity relations of melatonin and related indoleamines

AU Heward, Christopher B.; Hadley, Mac E.

CS Dep. Cell Dev. Biol., Univ. Arizona, Tucson, Ariz., USA

SO Life Sci. (1975), 17(7), 1167-77

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AB In vitro studies of the skin-lightening activity of melatonin (I) [73-31-4] and related indoleamines were conducted on the frog, *Rana pipiens*. Melatonin consistently produced lightening of skins at concns. as low as 5 times. 10⁻¹¹M. Other indoleamines having skin-lightening activity were ranked in approx. order of potency relative to melatonin, the most potent of the compds. studied. Studies of these and a no. of related, but inactive, compds. provided evidence suggesting structural requirements for compds. having activity on the melatonin receptor. Among the least potent of the active compds., a melatonin blocking agent, N-acetylserotonin [1210-83-9], was discovered. This prompted the synthesis of a second melatonin blocking agent, N-acetyltryptamine [1016-47-3], which, unlike N-acetylserotonin, had no intrinsic skin-lightening activity. The mechanism of blockade probably is by competitive inhibition at the binding site of the receptor. The intrinsic activity of indolic compds. on the melatonin receptor is detd. primarily by the moiety substituted on the 5th C, whereas, the affinity for the receptor binding site is detd. primarily by the moiety substituted on the 3rd C of the indole nucleus. Criteria for the identification of similar melatonin receptors in other tissues are suggested.

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IT 50-67-9, biological studies 54-16-0 61-54-1 73-31-4 87-51-4
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1019-45-0 1210-83-9 2208-41-5 3189-13-7 3471-31-6 3589-73-9

RL: BAC (Biological activity or effector, except adverse); BIOL
(Biological study)
(melanocyte response to)

STRUCTURE-ACTIVITY RELATIONSHIPS OF MELATONIN AND RELATED INDOLEAMINES

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(Received in final form September 9, 1975)

Summary

In vitro studies of the skin-lightening activity of melatonin and related indoleamines were conducted on the frog, *Rana pipiens*. Using objective, photometric measurements of reflectance, the response of the dermal melanocytes to these compounds was determined. Melatonin consistently produced significant lightening of skins at concentrations as low as 5×10^{-11} M. Other indoleamines found to possess skin-lightening activity were ranked in approximate order of potency relative to melatonin, the most potent of the compounds studied. Studies of these and a number of related, but inactive, compounds provided evidence suggesting structural requirements for compounds having activity on the melatonin receptor. Among the least potent of the active compounds, a melatonin blocking agent, N-acetylserotonin, was discovered. This prompted the synthesis of a second melatonin blocking agent, N-acetyltryptamine, which, unlike N-acetylserotonin, was shown to possess no intrinsic skin-lightening activity. The data suggest that the mechanism of blockade is by competitive inhibition at the binding site of the receptor. In addition, the data indicate that the intrinsic activity of indolic compounds on the melatonin receptor is determined primarily by the moiety substituted on the 5th carbon atom, whereas, the affinity for the receptor binding site is determined primarily by the moiety substituted on the 3rd carbon atom of the indole nucleus. Based on these findings, criteria for the identification of similar melatonin receptors in other tissues are suggested.

Indoleamines and their derivatives make up a large family of compounds widely distributed in nature. In the past two decades, increasing numbers of these compounds have been shown to occur naturally in mammalian systems (1, 2). Of these, most are synthesized in the brain and pineal gland from a common precursor, L-tryptophan (1,2). The metabolism of L-tryptophan and indoleamine biosynthesis has been discussed in detail elsewhere (1-3). Particular emphasis has been placed on the biosynthesis and metabolism of melatonin within the pineal gland. Stemming from this early biochemical data, a great deal of evidence has been generated suggesting possible physiological roles for melatonin in mammals (1,2,4); yet very little is

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known about the mechanism of action of this controversial indoleamine.

The present studies were initiated in order to characterize the melatonin receptor associated with the dermal melanocytes of the frog, *Rana pipiens*. Characterization of this receptor may provide a means for the identification of similar melatonin receptors in mammalian tissues. This, in turn, may lead to greater understanding of the mechanism of action of melatonin in mammals. The amphibian melanocyte was chosen as the model system for three reasons. First, the ability of physiological concentrations of melatonin to lighten amphibian skin had been previously well established (5,6). Second, the specificity, some structural requirements, and the relative potency of certain indoles on an amphibian system (i.e. *Xenopus laevis* larvae) had been previously demonstrated (7,8). Finally, the frog skin bioassay provided an objective and highly sensitive technique for measuring melatonin activity.

Materials and Methods

Compounds Studied. The indoleamines or related compounds (obtained from Sigma Chemical Company) used in these studies were: melatonin (N-acetyl-5-methoxytryptamine), 6-methoxyharmalan, 5-methoxytryptamine, 5-methoxyindole-3-acetic acid, 6-methoxyindole, 5-methoxy-D-L-tryptophan, N-acetylserotonin, (N-acetyl-5-hydroxytryptamine) 5-methoxy-N-N-dimethyltryptamine, 5-methoxyindole, N-acetylserotonin, harmine, tryptamine, 5-hydroxyindole-3-acetic acid, indole-3-acetic acid, and serotonin (5-hydroxytryptamine). Refer to Fig. 1 for the structures of these compounds. Porcine α -melanocyte stimulating hormone (α -MSH, obtained from Drs. Lande and Lerner) was used as the melanin granule dispersing agent in all experiments.

N-acetyltryptamine Synthesis. Since N-acetyltryptamine was not available commercially, the following method for the synthesis of this compound was adapted from Ho *et al.* (9). A mixture of 2 grams (10 millimoles), an equivalent amount of acetyl chloride, 12 millimoles of triethylamine, and 50 ml of chloroform was stirred at room temperature for approximately three hours. An equal volume of water was added, and the organic layer was separated and washed successively with 50 ml of 10% HCl, 50 ml of 2N NaOH, and 50 ml of water. After being dried with anhydrous sodium sulfate, the chloroform was evaporated *in vacuo*, and the solid product was recrystallized from cyclohexane. Recrystallization gave approximately a 20% yield of product (M.P. 74°C).

Bioassay. *Rana pipiens* of both sexes from the vicinity of Sinaloa, Mexico, obtained from Southwest Scientific Supply Co., were used in this study. Frogs were sacrificed by decapitation and skins from each animal were immediately prepared for photometric reflectance studies following the methods of Shizume *et al.* (10) and Wright and Lerner (11). Four pieces of skin could be obtained from each frog, one piece from each thigh and one from each lower leg. The skin samples were placed on aluminum rings and secured in place by a second ring made of bakelite. Each skin was placed within a 50 ml beaker containing 20 ml of amphibian Ringer's solution. The indolic compounds or other agents to be tested were added as 0.2 ml aliquots to the medium bathing the skins. This resulted in a 100-fold dilution of the experimental solution to yield the final molar concentration desired. Since most indoleamines are not readily dissolved in water all compounds were first dissolved in 1 ml of ethanol. Then water was added to yield the desired molar concentration. The initial average reflectance value for each group of skins was taken as the base value (100%) and all succeeding average values for each group were recorded as percent changes from the initial value. Percentage changes in reflectance were plotted against time. Changes in skin reflectance result from the movement of

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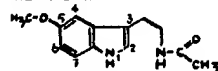
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COMPOUNDS FOUND TO HAVE FROG SKIN LIGHTENING ACTIVITY

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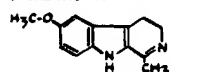
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6-HYDROXYMELATONIN

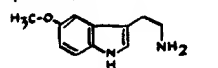


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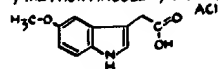
6-METHOXYHARMALAN

(10⁻⁴)

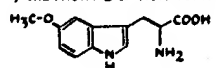
5-METHOXYTRYPTAMINE

(10⁻⁴)

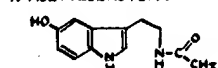
5-METHOXYINDOLE-3-ACETIC ACID

(10⁻⁵)

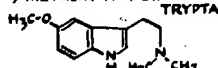
5-METHOXY-D-L-TRYPTOPHAN

(10⁻⁶)

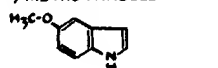
N-ACETYLSEROTONIN

(10⁻⁶)

5-METHOXY-N,N-DIMETHYL-TRYPTAMINE

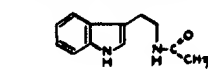
(10⁻⁷)

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(10⁻⁷)

COMPOUNDS FOUND TO HAVE NO FROG SKIN LIGHTENING ACTIVITY

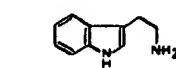
N-ACETYLTRYPTAMINE



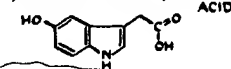
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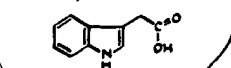
TRYPTAMINE



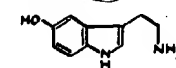
5-HYDROXYINDOLE-3-ACETIC ACID



INDOLE-3-ACETIC ACID



SEROTONIN

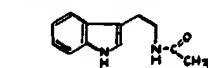


COMPOUNDS FOUND TO HAVE MELATONIN BLOCKING ACTIVITY

N-ACETYLTRYPTAMINE



N-ACETYLTRYPTAMINE



N-ACETYLSEROTONIN

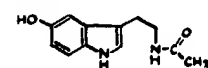


FIG. 1

Structural formulae of all compounds studied. Active lightening agents (left column) are ranked in order of potency relative to melatonin (numbers in parentheses). Inactive compounds (center column) are placed near active compounds of similar structure to facilitate comparison. Melatonin blocking agents (right column) are similarly located.

specific pigment granules within the dermal chromatophores of the frog skins (12). Light-absorbing melanin granules within melanocytes disperse and light-reflecting crystals within iridophores aggregate in response to MSH. This results in decreased reflectance (i.e. darkening) of the skins. Melatonin, on the other hand, causes aggregation of melanin granules within melanocytes but is without effect on iridophores. This results in increased reflectance (i.e. lightening) of skins. Because of the differential response of the dermal chromatophores, MSH darkened skins do not relighten to 100% of their original reflectance value upon addition of a maximal dose of melatonin (i.e. 10^{-9} M; Fig. 2).

Experimental Design. In each experiment the same basic format was generally utilized. The skins were initially darkened with α -MSH then, after 30 minutes, the experimental solutions were added and the response of the skins over time was observed. Reflectance readings were taken at regular intervals until no further significant change in reflectance was observed in two successive readings.

Statistical Analysis. Analysis of variance and the Student t-test were both applied in all experiments to determine the significance of any observed difference between groups. Both statistical methods yielded essentially identical levels of significance for all data reported.

Results

Sensitivity of Bioassay. The frog skins utilized in this study were found to be extremely sensitive to melatonin. Significant skin lightening activity was observed consistently with melatonin concentrations of 5×10^{-11} molar. Lightening activity was also observed at melatonin concentrations of 10^{-11} molar, but the effects were not always statistically significant (Fig. 2).

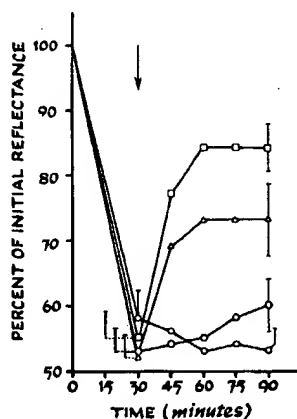


FIG. 2

Lightening response of MSH-darkened *Rana pipiens* skins to three different concentrations of melatonin. Four groups of skins were darkened with MSH (5×10^{-10} g/ml). Then, at 30 min (\downarrow), melatonin was added to three of the groups at the following concentrations: 10^{-9} M (□), 10^{-10} M (Δ), 10^{-11} M (○). As controls, one group of skins (○) was allowed to remain in MSH throughout the experiment. Results are means of the reflectance measurements from the eight skins in each group. Standard errors of selected means are shown.

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A dose response could be obtained only within a narrow range of melatonin concentrations very near the minimum effective dose (i.e. 10^{-11} molar). Doses higher than 10^{-9} molar usually caused maximal lightening of the skins. Note that skins do not relighten to their original reflectance value after addition of melatonin.

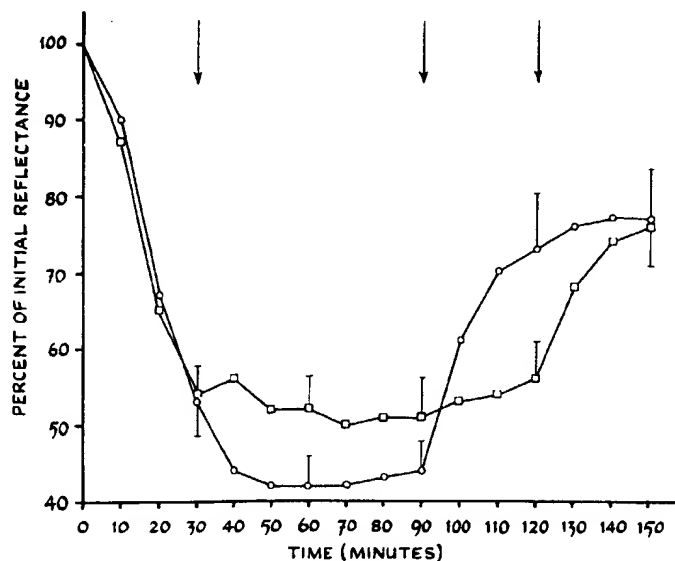


FIG. 3

Response of MSH-darkened *Rana pipiens* skins to N-acetyl-5-hydroxytryptamine and subsequent blockade of the melatonin lightening response. After the initial base photometric reading, MSH (5×10^{-10} g/ml) was added to both groups of skins. Then, after 30 min (\downarrow) N-acetyl-5-hydroxytryptamine (\square) was added to one group of skins. The other group of skins (O) was maintained as a control. At 90 min (\downarrow) melatonin (10^{-10} M) was added to both groups of skins; at 120 min (\downarrow) melatonin (10^{-8} M) was again added to both groups. Results are means of the reflectance measurements from eight skins for each point on the graph. Standard errors of selected means are shown.

Specificity of Receptor. Although several other compounds were found capable of eliciting the melanin aggregating response, none of the compounds tested possessed a potency equal to that of melatonin. Only 6-hydroxymelatonin, with a minimum effective dose of 10^{-10} molar, had a potency comparable to that of melatonin, with a minimum effective dose of 5×10^{-11} molar. The minimum effective dose for the next three most potent structural analogs of melatonin was 10^{-7} molar. Thus, using this criterion melatonin is less than 10 times more potent than 6-hydroxymelatonin but 10,000 times more potent than the next three most potent compounds in lightening the frog skins.

Those compounds having skin lightening activity were ranked in approximate order of decreasing potency. The compounds are: melatonin (1),

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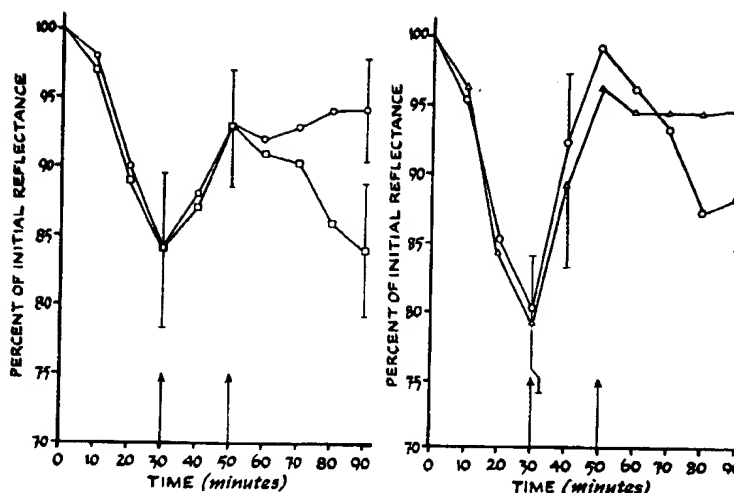


FIG. 4

Response of MSH-darkened *Rana pipiens* to melatonin and subsequent darkening by N-acetylserotonin. After the initial base photometric reading, MSH (5×10^{-10} g/ml) was added to all groups of skins. Then, after 30 min (\uparrow) melatonin at concentrations of 10^{-10} M (\square & \square) and 10^{-9} M (Δ & \circ) was added to the skins. Then at 50 min (\uparrow), N-acetylserotonin (10^{-4} M) was added to two groups of skins (\square & \circ). Results are means of the reflectance measurements from eight skins for each point on the graph. Standard errors for selected points are shown.

6-hydroxymelatonin (≈ 1), 6-methoxyharmalan (10^{-4}), 5-methoxytryptamine (10^{-4}), 5-methoxyindole-3-acetic acid (10^{-5}), 5-methoxy-D-L-tryptophan (10^{-6}), N-acetylserotonin (10^{-6}), 5-methoxy-N,N-dimethyltryptamine (10^{-7}), 5-methoxyindole (10^{-7}). Numbers in parentheses represent the approximate potency of each compound relative to melatonin (Fig. 2).

Several compounds were tested which showed no skin lightening activity at the concentrations used. They are: N-acetyltryptamine, tryptamine, harmine, 5-hydroxy-indole-3-acetic acid, indole-3-acetic acid, and serotonin. Conceivably, some of these compounds may have some lightening activity at concentrations higher than those used in these experiments. The highest concentration at which any of these compounds was tested was 10^{-4} molar.

Melatonin Blocking Agents. Of the compounds tested, two were found to be capable of inhibiting the skin lightening activity of melatonin. N-acetylserotonin, the only active lightening compound not possessing a methoxy group, was found to block melatonin very effectively. However, the intrinsic lightening activity of this compound tended to mask its melatonin blocking activity (Fig. 3). The ability of a higher concentration of melatonin to override the blockade suggests that the blockade is competitive in nature. In addition, the ability of N-acetylserotonin to darken skins previously lightened with melatonin provides further evidence that the mechanism of blockade is by competition for the melatonin receptor (Fig. 4).

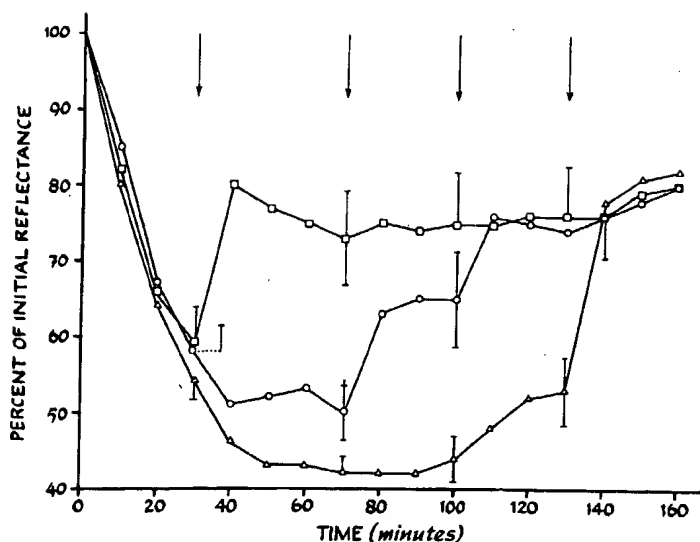


FIG. 5

In *vitro* demonstration of N-acetyltryptamine blockade of melatonin on *Rana pipiens* skins. Three groups of skins were darkened with MSH (5×10^{-10} g/ml). Then, at 30 min (\downarrow) 10^{-4} M melatonin (\square) and 10^{-4} M N-acetyltryptamine (Δ) were each added to both groups of skins. One group of skins (O) was maintained as a control. At 70 min (\downarrow), 100 min (\downarrow), and 130 min (\downarrow), melatonin at concentrations of 10^{-10} M, 10^{-8} M and 10^{-6} M, respectively was added to all groups of skins. Results are means of the reflectance measurements from seven skins for each point on the graph. Selected standard errors are provided.

N-acetyltryptamine was synthesized in order to test our hypothesis that removal of the 5-hydroxy group from N-acetylserotonin would abolish the intrinsic lightening activity of the molecule without reducing its melatonin-blocking activity. This hypothesis was experimentally confirmed (Fig. 5). The tryptamine from which the N-acetyltryptamine was synthesized possessed neither intrinsic lightening activity nor melatonin-blocking activity when tested on the skins.

N-acetyltryptamine blocked melatonin induced lightening of MSH-darkened skins whether it was added to the skins prior to, simultaneously with, or after the addition of melatonin. This blocking activity could be reversed by addition of a 10-fold higher concentration of melatonin, further demonstrating the competitive nature of the blockade.

Discussion

Since melatonin (N-acetyl-5-methoxytryptamine) was first isolated from bovine pineal tissue in 1959 (5) a great deal of controversy has arisen concerning the endocrine properties of this indole. Evidence exists

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indicating that melatonin exerts some influence on a wide range of physiological processes, including: central nervous system activity (13,14), pituitary function (15,16), adrenal function (17), thyroid function (18,19) and the activity of reproductive systems (20, 21). The selective uptake of administered melatonin by certain endocrine tissues has been used as evidence supporting a hormonal action on these systems (22). However, most of the effects observed in these systems were achieved using melatonin concentrations approaching pharmacological levels. Thus, one must wonder whether the effects observed on these systems represent naturally occurring physiological phenomena or simply the pharmacological consequences of melatonin toxicity.

The present approach toward resolving this question involves the characterization of a melatonin receptor in a system known to respond to melatonin at physiological concentrations. When enough characteristics of the receptor are known, criteria can be established for the identification of similar receptors in other tissues. For example, analogous criteria are now widely used for the identification of receptors for catecholamines (23). If melatonin receptors can be identified in a particular tissue, then the hypothesis that the tissue in question is a target tissue for melatonin *in vivo* is strongly supported. If, on the other hand, no such receptors can be found, then the evidence suggests rejection of that hypothesis.

The results of many experiments are summarized in Fig. 1. Based on these data, certain observations can be made concerning the structural requirements for activity on the melanocyte melatonin receptor. First, it is clear that the presence of a methoxy group on the 5th carbon atom is essential for the skin lightening activity of the active compounds. All but one of the active compounds had a methoxy group on the 5th carbon atom of the basic indole nucleus. Compounds identical to certain active compounds, except for lacking the 5-methoxy group, had no measurable lightening activity (e.g. melatonin vs. N-acetyltryptamine, 5-methoxytryptamine vs. tryptamine).

The second observation concerns the structural requirements for effective binding of a compound to the melatonin receptor. These requirements are best exemplified by those compounds shown to have a high affinity for the receptor. Melatonin, obviously, has the highest receptor-affinity of all the active compounds, hence its great potency. Comparison of the structures and relative potencies of melatonin and 5-methoxytryptamine suggests that the N-acetyl group plays an important role in receptor binding. Consistent with this hypothesis is the fact that both of the melatonin blocking agents possess the N-acetyl group in the same position as melatonin. Clearly, these compounds have a receptor affinity comparable to that of melatonin, hence their ability to compete with melatonin for the receptor binding site. The proposed structural requirements of the melatonin receptor of frog skin melanocytes in terms of binding site affinity and intrinsic agonistic activity are shown in Fig. 6.

These observations on structure-activity relationships of melatonin and related indoleamines are in general agreement with the previous study of Quay (8). Our results reveal a structurally narrow requirement for melatonin antagonism of MSH action. The tryptamine nucleus and the 5-methoxy and N-acetyl groups are essential for complete activity. We found, however, that 6-hydroxylation of melatonin only slightly diminished (if at all) the effectiveness of melatonin. Quay (8), using *Xenopus laevis* larvae, and Finnin and Reed (24), using a pencil fish bioassay, found that this hydroxylation raised the minimum effective dose approximately 10^3 times. In addition, these investigators found 6-methoxyindole to be a relatively potent lightening agent. Under our experimental conditions, however, this

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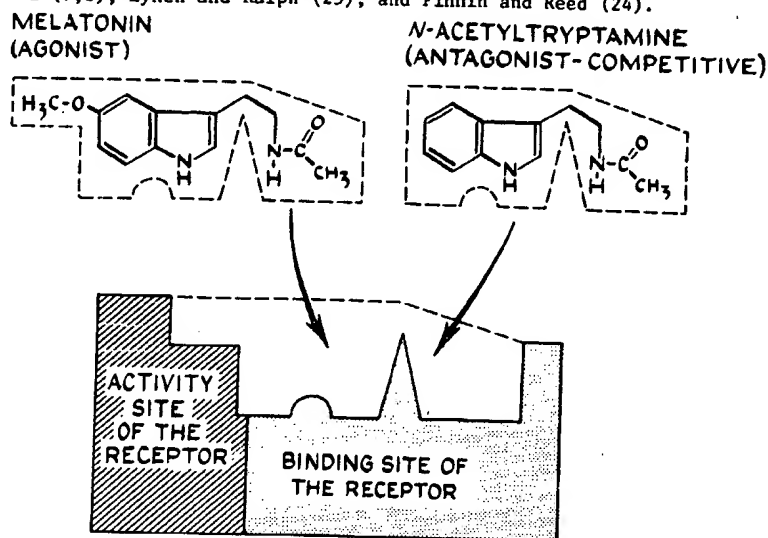
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compound was insoluble and, thus, had no skin-lightening activity. The *in vitro* frog skin bioassay provides an objective quantitative method for estimating the potency of melatonin and related compounds and may be generally preferable to the subjective *in vivo* methods of Quay and Bagnara (7,8), Lynch and Ralph (25), and Finnin and Reed (24).



MELATONIN RECEPTOR

FIG. 6

Schematic representation of proposed compound-receptor interactions demonstrating the structural requirements for agonistic and antagonistic activity on the melatonin receptor. Note that the 5-methoxy group is essential for intrinsic activity on the receptor, whereas, the moiety attached to the 3rd carbon atom of the indole nucleus, including the N-acetyl group, help determine the affinity of the compounds for the binding site of the receptor.

A third observation provides additional confirmation of the structural requirements discussed above and suggests a direction for further research which may lead to the discovery of compounds with greater lightening activity than melatonin. N-acetylserotonin is the only lightening agent having no methoxy group at all. Presumably the 5-hydroxy group of this compound is responsible for its lightening activity. Serotonin, on the other hand, has no lightening activity. Apparently, the N-acetyl group allows the N-acetylserotonin molecule to bind to the receptor in exactly the right position to allow the 5-hydroxy group to exert some effect. Perhaps other functional groups, properly placed on the 5th carbon atom of N-acetyltryptamine would have a greater potency than the methoxy group itself. To test this hypothesis, other compounds such as N-acetyl-5-chlorotryptamine and N-acetyl-5-isopropoxytryptamine should be synthesized and their skin-lightening activity determined. Conversely, substitutions on the N-acetyl moiety may lead to the production of more potent melatonin antagonists. Preliminary studies of the agonistic activity of several compounds representing these two types of substitutions revealed no compound more potent than melatonin (26). The melatonin blocking activity of these compounds was not

studied. Further research on these and related structures will be initiated in our laboratory.

In view of the growing evidence for melatonin effects in mammalian endocrine tissues and the possible existence of specific melatonin receptors in these tissues, the need for a method of identifying such receptors is clear. Toward this end the author's propose the following tentative definition of a melatonin receptor:

A melatonin receptor is one which mediates a response pharmacologically characterized by:

- 1) A relative potency series in which melatonin >> 5-methoxytryptamine > 5-methoxyindole > serotonin; and
- 2) A susceptibility to specific blockade by N-acetyltryptamine at relatively low concentrations.

It should be mentioned that the first criterion may be difficult to meet in intact animals due to the possible enzymatic conversion of 5-methoxytryptamine, 5-methoxyindole, and serotonin to melatonin *in vivo*. In such systems, perhaps, greater emphasis should be placed on meeting the second criterion.

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